Journal of Chromatography, 576 (1992) 263–270
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6272

High-performance liquid chromatographic determination of selenocysteine with the fluorescent reagent, N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid

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(First received September 3rd, 1991; revised manuscript received January 7th, 1992)

ABSTRACT

The method described is based on derivatization of selenocysteine with N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid and responds linearly to selenocysteine spiked into plasma. Recovery is insensitive to inter-individual variation or use of serum *versus* plasma, but is decreased by hemolysis. The derivative is stable for at least three days. The total imprecision of determinations in plasma was 0.8–2.1% (coefficient of variation) over the range of 6–30 μM selenocysteine, with a detection limit of 0.4 μM (3 × S.D.). There was no significant interference from plasma thiols. This appears to be the first report of the selective reaction of free selenocysteine with a fluorescent reagent. This simple method works well in plasma and serum and may be adaptable to other types of samples.

INTRODUCTION

Selenocysteine (SeCys), the selenium-containing analog of cysteine (Cys) in which selenium (Se) replaces sulfur, occurs in plants [1], bacteria [2] and animals [3] and is at the active sites of all well characterized selenium-requiring enzymes in bacteria and animals [4]. SeCys in proteins was the only form of selenium found in rats fed a diet containing selenium as sodium selenite [5]. Rat liver contains a SeCys-specific transfer RNA [6] which inserts intact SeCys into the polypeptide backbone of glutathione peroxidase during protein synthesis [7]. The SeCys transfer RNA is ubiquitous throughout the animal kingdom [8] and uses the opal termination codon, UGA, to incorporate SeCys into glutathione peroxidase [9], formate dehydrogenase [4], glycine reductase [10], hydrogenase [4] and plasma "selenoprotein P" [11]. Serine is initially aminoacylated to the SeCys-specific transfer RNA and subsequently converted to SeCys while still attached to the transfer RNA [4,11]. While the mechanisms by which the ribosome discriminates a UGA termination codon from a UGA SeCys codon have not been fully elucidated, SeCys can be considered the 21st protein amino acid [4].

The methods used to determine SeCys have generally relied upon amino acid analyzer columns to separate SeCys (or a derivative) and ^{75}Se radioactivity to detect the SeCys [12,13]. Most methods have been developed for SeCys in protein, using either derivatization of the selenium [13], anaerobic hydrolysis [14] or enzymatic digestion [3] to obtain the labile SeCys as the free amino acid. Only one method has been reported for determining free SeCys, which also relied upon a ^{75}Se tracer and amino acid analysis of the Se-carboxymethyl derivative formed by reaction with iodoacetic acid [7]. While it is clear that SeCys in protein is synthesized on the transfer RNA, significant amounts of free [^{75}Se]SeCys

were nevertheless detected in rat liver slices incubated with sodium [^{75}Se]selenite [7]. The physiological significance of free SeCys in animals is uncertain, but in selenium accumulator plants it is apparently a storage form of selenium [1].

We report here what we believe to be the first method for determination of free SeCys based upon selective derivatization of the selenium with a fluorescent reagent. The reagent, N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (IAEDANS), reacts preferentially with SeCys relative to Cys at acid pH, minimizing potential interferences from thiols. This simple method exhibits a high recovery of and linear response to added SeCys and should be useful for studying the metabolism of this amino acid.

EXPERIMENTAL

Materials and instrumentation

Sodium citrate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Trolox was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) was performed with a Perkin-Elmer Series 4 quaternary solvent delivery system and ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, USA) using an FS-970 fluorescence detector (Applied Biosystems, Foster City, CA, USA). Peak heights were calculated with an Omega 4 computerized chromatography data system (Perkin-Elmer, Norwalk, CT, USA). All chemicals used were of reagent grade or higher quality.

Derivatization procedure

Urea (1 g) was dissolved in 1 ml plasma or serum (with or without selenocystine spike). To 0.5 ml of the mixture were then added 5 μl of 2-octanol (to inhibit foaming) and 50 μl of 1.65 *M* KBH_4, 8 *M* urea and held for 30 min at room temperature to reduce oxidized forms of SeCys to the free selenol form. After reduction, a total of 0.25 ml of 1 *M* NaH_2PO_4, 2 m*M* Na_2EDTA, pH 3.0 was added in two portions with vigorous mixing (vortex mixer) after each addition to minimize foaming (final pH 6.6). Immediately thereafter, 50 μl of freshly prepared 3.4 m*M* IAEDANS in dimethylformamide were added and mixed thoroughly and the reaction was allowed to proceed for 15–60 min at room temperature. Since IAEDANS is subject to photodegradation [15], the reagent, reaction mixtures and derivatized samples were protected from prolonged exposure to bright light. The reaction was stopped by addition of 1.15 ml of 1.06 *M* trichloroacetic acid, 0.6 *M* hydrochloric acid. The samples were left on ice for at least 20 min, the precipitates were removed by centrifugation (15 min at 3000 *g*, 5°C) and the supernatants were filtered through Acro LC-13 filters (Gelman Sciences, Ann Arbor, MI, USA) into 2-ml glass sample vials. Repeated analyses of prepared samples stored at room temperature for several days showed that the filtered samples were stable for at least three days.

Chromatographic separation

A 5–50 μl volume (standard injection volume = 20 μl) of the filtered trichloroacetic acid supernatants were injected onto an Ultrasphere ODS column (250 mm × 4.6 mm I.D., 5 μm particle size; Beckman Instruments, Fullerton, CA, USA) fitted with a guard column (RP-18 cartridge, 30 mm × 4.6 mm I.D., 5 μm; Brownlee Labs., Santa Clara, CA, USA) at ambient temperature (22 ± 4°C). The mobile phase was an 8:2 (v/v) mixture of solution A (50 m*M* formic acid, 60 m*M* acetic acid, adjusted to pH 5.1 with sodium hydroxide) and solution B (solution A–isopropanol, 97:3). The flow-rate was 1.5 ml/min. Fluorescence detection was accomplished by excitation at 315 nm and measurement of emission using a 418-nm cut-off, high-pass filter. At 20 min into the run (after elution of the SeCys-AEDANS derivative) a 3-min gradient to 90% solution B and a 10-min hold at 90% solution B were used to elute fluorescent by-products of the derivatization reaction. A 3-min gradient back to the initial conditions and a 10-min re-equilibration period prepared the column for the next sample injection. A complete gradient was run before each series of samples to condition the column. Samples were

subsequently injected approximately every 46 min.

Other assays

Selenium was determined by the fluorometric method of Watkinson [16]. Fluorescence spectra were measured with an Aminco-Bowman ratio spectrofluorimeter (American Instrument, Silver Spring, MD, USA). The production of SeCys (selenol form) from the reduction of selenocystine (diselenide form) was assessed by measurement of the SeCys with 5,5′-dithiobis(2-nitrobenzoic acid) [17].

RESULTS

Fluorogenic reagents

Since the original goal of this research was to develop a non-chromatographic, fluorescence-based method for the determination of SeCys in the presence of thiols such as Cys, we investigated the spectral characteristics of the fluorescent derivatives formed by reaction of SeCys and Cys with reagents that were not initially fluorescent. Monobromobimane [18], *o*-phthalaldehyde [19], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole [20], 4-chloro-7-nitrobenzofurazan [21], 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate [22], 4-chloro-7-sulfobenzofurazan [23], N-1-pyrene-maleimide [24] and N-(7-dimethylamino-4-methylcoumaryl) maleimide [25] showed no significant differences between the fluorescence spectra of their SeCys and Cys derivatives. Therefore differences between the reactivities of SeCys and Cys with IAEDANS and chromatographic separation of their derivatives were used to develop the present method for the specific fluorescence-based determination of SeCys.

Chromatographic conditions

The SeCys-AEDANS derivative eluted at 19 min in the isocratic portion of the chromatogram (Fig. 1). A small, unidentified peak at 19.8 min eluted immediately after the SeCys-AEDANS derivative peak in all chromatograms. Although a variety of chromatographic conditions and mobile phase compositions were tested, none were found to improve the resolution between the SeCys-AEDANS derivative and the 19.8-min peak. Since the slight overlap between these peaks interfered with peak-area measurements, the amount of SeCys-AEDANS derivative was quantitated based on the peak height. Although the elution time of the SeCys-AEDANS derivative was sensitive to temperature, decreasing by 30% upon a 10°C temperature rise, resolution from potentially interfering peaks was not adversely affected by small temperature changes.

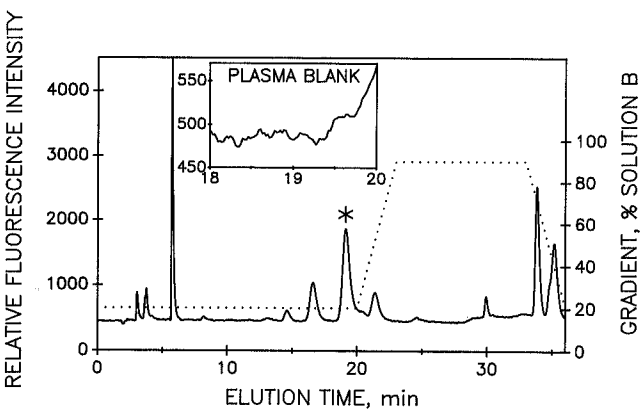

Fig. 1. Typical chromatograms of the SeCys-AEDANS derivative. SeCys (12 μM) was derivatized with IAEDANS in a plasma matrix and 20 μl of the trichloroacetic acid supernatant were chromatographed as described in the Experimental section. The asterisk marks the SeCys–AEDANS peak at 19 min. The dotted line indicates the solvent composition as the percentage of solution B (3% isopropanol in solution A). The inset shows an expanded view of the baseline around the elution position of the SeCys-AEDANS derivative for a 20-μl injection of the trichloroacetic acid supernatant from the derivatization of a plasma sample without added SeCys.

Chromatography was therefore conducted at ambient temperature (22 ± 4°C). No degradation of column performance was observed during several hundred sample injections.

Optimization of reaction conditions

Since SeCys is easily oxidized in air and only the fully reduced selenol form reacts readily with haloalkyl reagents such as IAEDANS, it was first necessary to insure that the SeCys in the sample was present as the selenol. Selenocystine, the diselenide form of SeCys, was used as a model of the oxidized forms of SeCys that might be present in real samples. The reduction of selenocystine to SeCys was complete within 15 min (data not shown), however, a reduction time of 30 min was chosen to allow time for the reduction of other oxidized forms of SeCys.

The rate of reaction of IAEDANS with SeCys and Cys was dependent on the pH of the reaction mixture (Fig. 2). A poly buffer system of 0.1 *M* citrate, 0.1 *M* phosphate, 0.17 *M* borate, 3 m*M* EDTA was used to vary the reaction pH over the range 2–12. At pH 9.4, the reaction rate with Cys was half of that obtained at pH 12 and the reaction rate with SeCys was half-maximal at pH 4.3, suggesting that the selective reaction of SeCys with IAEDANS would be favored at acid pH.

To assess the specificity advantage in favor of reaction with SeCys that might be obtainable by manipulating the reaction pH, mixtures of SeCys and Cys were reacted with IAEDANS at pH 2.2–5.5 and the derivatives were quantitated by HPLC. The separation of the SeCys-AEDANS and Cys-AEDANS derivatives using the recommended chromatographic procedure is illustrated in Fig. 3. The SeCys-AEDANS derivative peak height and the Cys-AEDANS derivative peak height were used to calculate apparent first-order rate constants for each of the competing reactions at each pH. The ratio of the SeCys rate constant to the Cys rate constant at each pH represented the specificity factor in favor of reaction of SeCys with IAEDANS. Fig. 4 shows that the specificity factor increased with decreasing pH, with the highest value observed at pH 2.2. The apparent linear relationship between the logarithm of the specificity factor and pH (inset to Fig. 4) suggested that even greater specificity for SeCys could be obtained at a pH below 2.2. However, the yield of SeCys-AEDANS derivative declined with decreasing pH, even when the reac-

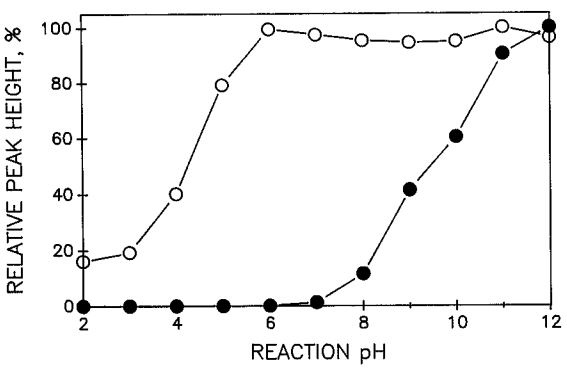

Fig. 2. Effect of pH on the reaction of IAEDANS with SeCys and Cys. After borohydride reduction, 1 m*M* SeCys (open circles) or 1 m*M* Cys (filled circles) were reacted separately without a sample matrix for 1 min with 0.05 m*M* IAEDANS in 0.1 *M* citrate, 0.1 *M* phosphate, 0.17 *M* borate, 3 m*M* EDTA, pH 2–12 under nitrogen and 5 μl of the acid supernatant were injected and chromatographed as described in the Experimental section. The derivative peak heights were normalized such that, for each derivative, the highest observed peak height equaled 100%.

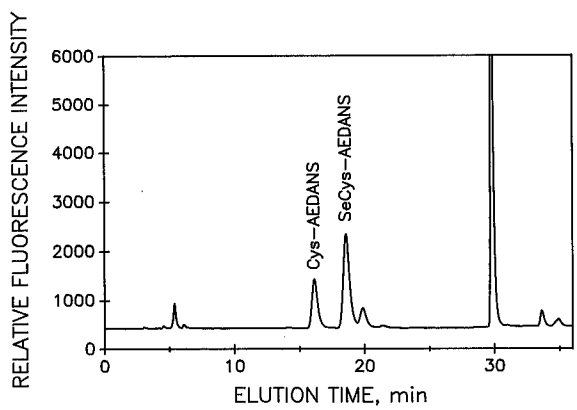

Fig. 3. Separation of the SeCys-AEDANS derivative from the Cys-AEDANS derivative. After borohydride reduction, a mixture of 150 μ*M* SeCys and 175 μ*M* Cys was derivatized with IAEDANS in the absence of a sample matrix. In order to increase the reaction rate with Cys, the reaction pH was increased to pH 10.1. Other reaction and chromatographic conditions were as described in the Experimental section. A 5-μl aliquot of acid supernatant was injected.

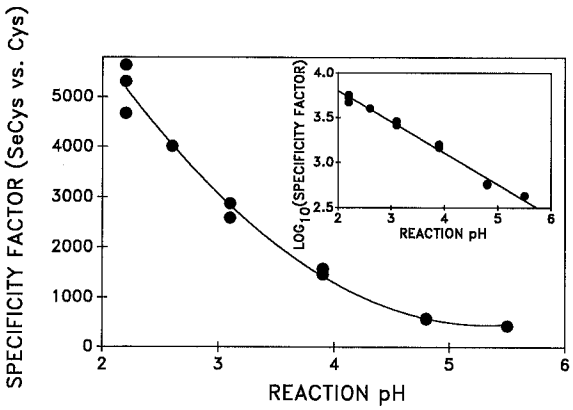

Fig. 4. Effect of pH on the specificity of IAEDANS reaction with SeCys. After borohydride reduction, a mixture of 0.25 m*M* SeCys and 125 m*M* Cys was reacted without a sample matrix for 1 min with 2.5 m*M* IAEDANS in 0.1 *M* citrate, 0.1 *M* phosphate, 0.17 *M* borate, 3 m*M* EDTA, pH 2.2–5.5 under nitrogen and chromatographed as described in the Experimental section. The derivative peak heights were used to estimate the reaction rate of IAEDANS with each amino acid at each pH. The specificity factor was calculated as the ratio of the apparent first-order rate constant for reaction with SeCys to the apparent first-order rate constant for reaction with Cys. The inset shows the apparent linear dependence of the logarithm of the specificity factor on pH.

tion time was increased to compensate for the decreased reaction rate. A pH of 6 was chosen as the optimal reaction pH because the reaction rate of IAEDANS with SeCys was maximal at pH 6 whereas the reaction of IAEDANS with Cys was barely discernible at this pH (Fig. 2). In practice, the phosphate buffer used in the recommended procedure resulted in a pH of 6.6, which proved to be adequate for this application. Studies of the time course of the reaction of IAEDANS with SeCys in plasma at pH 6.6 (data not shown) showed the reaction was essentially complete within 10 min and that there was no increase in yield at 60 min beyond that obtained at 15 min. A reaction time of 15 min was judged adequate to ensure a complete reaction of SeCys with IAEDANS at pH 6.6.

Interferences

The type of sample matrix affected the yield of SeCys-AEDANS derivative from samples spiked with selenocystine (Table I). Plasma and serum samples were found to give the highest yields of SeCys-AEDANS derivative. There was no signif-

TABLE I

FACTORS AFFECTING THE YIELD OF SeCys-AEDANS DERIVATIVE

Selenocystine spiked samples (26 μM SeCys) were taken through the complete procedure. Liver and erythrocytes were diluted to protein concentrations comparable to plasma. Additives (pH 6.6) were added after reduction. The percentage yield is relative to plasma without additives.

Additive	Final concentration in derivatization reaction	Recovery (%)		
		Plasma	Liver[a]	Erythrocytes[b]
None		100	78.7	10.8
Sodium citrate	0.1 *M*	98.8	85.6	14.3
EDTA	5 m*M*	96.9	80.1	12.4
Deferroxamine	25 m*M*	102.7	81.9	16.1
2,2′-Dipyridyl	5 m*M*	98.5	82.1	6.5
Trolox[c]	25 m*M*	101.1	83.3	13.3
Dithiothreitol	5 m*M*	107.4	84.1	35.9
Ascorbic acid	25 m*M*	103.6	85.1	12.9
Ascorbic acid	0.1 *M*	104.9	97.7	Not done
Hemoglobin	1.5 g/l	34.2	Not done	Not done

[a] Liver sample was a 1:2 homogenate of porcine liver–water.

[b] Erythrocyte sample was a 1:3 dilution of frozen human erythrocytes–water.

[c] Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, is a water-soluble analogue of tocopherol.

icant difference between the yields in plasma and serum, nor between plasma samples from different individuals. Replacement of the sample matrix with distilled water caused the yield of SeCys-AEDANS derivative to decrease to one third of the yield in plasma. The yield of SeCys-AEDANS derivative from spiked porcine liver homogenate samples was 21% lower than the yield in plasma, however, addition of 0.1 *M* ascorbic acid in the derivatization reaction increased the yield in liver samples almost to the level obtained in plasma. The yield of SeCys-AEDANS derivative was decreased by an average of 24% in plasma and serum samples which showed evidence of red cell hemolysis (not shown) and the yield in erythrocytes was decreased by about 89% relative to the yield in plasma or serum (Table I). Addition of purified hemoglobin to spiked plasma (Table I) indicated the role of this protein in causing the decreased yield in erythrocytes and hemolyzed plasma and serum. Various antioxidants and metal chelators added to the derivatization reaction caused slight increases in the yield of SeCys-AEDANS derivative in erythrocyte samples (Table I), but the yield was still greatly decreased compared to the yield in plasma.

Selenocysteamine was evaluated as an internal standard to correct for differences in the yield between different tissues. Selenocysteamine reacted readily with IAEDANS using the recommended procedure and the selenocysteamine-AEDANS derivative was well separated from the SeCys-AEDANS derivative and easily quantitated using the recommended chromatographic conditions. However, the internal standard did not normalize the differences in yield between tissues nor did it improve the precision within a tissue and it was therefore not used for subsequent analyses.

The fluorescent derivative formed by reaction of IAEDANS with glutathione eluted at the same time as the SeCys-AEDANS derivative, however, chromatograms of plasma derivatized without added selenocystine (inset to Fig. 1) did not show any evidence of a peak at the elution position of the SeCys-AEDANS derivative. A concentration of 1.25 m*M* glutathione was found to yield a peak height equivalent to that obtained from 12 μM SeCys, indicating that the sensitivity for SeCys was about 100 times greater than the sensitivity for glutathione. The low concentration of glutathione in plasma [26] explains the lack of significant interference from endogenous plasma glutathione at the elution position of the SeCys–AEDANS derivative. Derivatization reaction times up to 60 min did not increase the tendency of endogenous plasma glutathione to interfere in the determination of SeCys.

Standard calibration graph

Selenocystine was spiked into plasma samples at levels equivalent to SeCys concentrations of 6, 12, 18, 24 and 30 μM and carried through the complete reduction, derivatization and chromatography procedure to generate the daily calibration graphs. There was no evidence of non-linearity over this range of SeCys concentration and the correlation coefficient was always greater than 0.998. The regression intercepts in calibration graphs were not significantly different from zero, reflecting the lack of significant interference from a co-eluting peak due to endogenous plasma glutathione.

Precision and recovery

The within-day repeatability and between-day reproducibility of the method were evaluated by repeated derivatizations of replicate plasma samples spiked with graded levels of selenocystine and the data are shown in Table II. The within-day standard deviation for the lowest standard tested (1.5 μM) was used to estimate a limit of detection (3 × S.D.) of 0.4 μM SeCys in plasma, based on 20-μl injections.

To assess the absolute recovery of SeCys as the SeCys-AEDANS derivative, ten 50-μl injections of trichloroacetic acid supernatant from the derivatization of plasma spiked with selenocystine equivalent to 50 μM SeCys were chromatographed and the eluate was collected in 0.5-min (0.75-ml) fractions. Corresponding fractions from all ten injections were collected into the same set of test tubes and analyzed for their total selenium content. More than 95% of the seleni-

TABLE II

WITHIN-DAY AND TOTAL (DAY-TO-DAY) IMPRECISION OF SeCys DETERMINATIONS

Plasma samples spiked with selenocystine were taken through the complete procedure. The injection volume was 20 μl. Within-day imprecision was estimated from analysis of five plasma samples performed on the same day. Total (day-to-day) imprecision was estimated from analysis of five plasma samples performed on five separate days, one each day, over a one year period. The total (day-to-day) imprecision therefore includes the contribution from within-day sources of error.

SeCys concentration (μM)		Imprecision			
		Within-day		Total (day-to-day)	
Spiked	Found	S.D. (μM)	C.V. (%)	S.D. (μM)	C.V. (%)
1.5	1.75	0.14	7.7	Not done	
6	5.93	0.07	1.1	0.48	8.1
18	17.6	0.14	0.8	0.56	3.2
30	28.9	0.60	2.1	0.51	1.8

um in the fractions was recovered in a peak at 19-min elution time, where the SeCys-AEDANS fluorescent derivative eluted. The SeCys-AEDANS derivative peak accounted for 81% of the starting selenium, based on direct determination of the selenium concentration in the selenocystine spiking solution.

DISCUSSION

Although many fluorescent or fluorogenic derivatizing reagents are available for determination of thiols, IAEDANS was selected for this application because of previous work which found that SeCys continued to react readily with iodoacetic acid at pH values far below the pK_a of the SeCys selenol [27]. We observed a similar but smaller effect in the pH dependence of the reaction of SeCys with IAEDANS, which contains the iodoacetyl group as the reactive part of its structure. While the pK_a of the SeCys selenol is 5.24 [27], the half-maximal reaction rate of SeCys with IAEDANS was observed at pH 4.3. Since the lower pK_a of the SeCys selenol will confer some specificity relative to Cys with any electrophilic reagent and since fluorescent thiol derivatives formed by reaction with other reagents could presumably be separated from the SeCys derivatives by chromatography, it is likely that other thiol-specific fluorescent derivatizing reagents would also be useful for the selective derivatization of SeCys. This possibility deserves further investigation.

Since this method was developed using selenocystine as a model for endogenous oxidized forms of SeCys it is not certain that other oxidized forms of SeCys which may occur in biological samples would be detected with similar efficiency. However, borohydride is known to efficiently reduce many oxidized organic selenium compounds [28]. Furthermore, experiments to trap [^{75}Se]SeCys in rat liver slices by reaction with iodoacetic acid recovered little or no carboxymethyl[^{75}Se]SeCys unless the samples were reduced with borohydride [7], showing that at least some of the endogenous oxidized forms of SeCys can be reduced by borohydride.

Based on our results with porcine liver, this method may also be suitable for SeCys determinations in tissues other than plasma and serum, with the notable exception of erythrocytes. The fact that antioxidants and iron chelators increased the yield of SeCys-AEDANS derivative in erythrocyte samples (Table I) while hemolysis or the addition of hemoglobin decreased the yield in plasma samples suggests that hemoglobin, or iron released from hemoglobin, caused the SeCys to be re-oxidized after the borohydride reduction step. The greatly decreased yield of SeCys-AEDANS derivative in the absence of a sample matrix suggests that some component in plasma and serum protects the SeCys from re-oxidation, possibly the plasma proteins or the antioxidants, uric acid, ascorbic acid and bilirubin, which are abundant in plasma and serum [29]. Application of this method to other tissues may be limited by high concentrations of glutathione, which might cause chromatographic interference with the SeCys-AEDANS derivative peak at 19 min or by poor recoveries of the SeCys-AEDANS derivative.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Ms. LaVette Green for her assistance with the preparation of this manuscript. Mention of trade name, proprietary product of specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture, nor does it imply approval to the exclusion of other products that may be suitable.

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